Blocking CD44 Receptor Protein Stimulates Dedifferentiation in Chondrocytes

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Introduction and Background

Three-dimensional gels are an important tool in biological engineering because most cells are not found on two-dimensional surfaces in vivo. Therefore, to accurately replicate natural conditions, it is important to create materials that can emulate the environment cells are accustomed to. Hydrogels have stepped in as the prime conduit by which experiments in three dimensions are carried out. More specifically, Alginate gels provide scaffolding representative of natural conditions for chondrocytes and can also be easily be adjusted to have different diffusive properties and therefore can simulate various conditions. However, they are not perfect; our goal in this study was to find some way to limit the dedifferentiation of chondrocytes back into fibroblasts in alginate gels. This is a big problem, because the end goal is to create a material that is suitable for the culturing of chondrocytes without affecting their normal morphology.

In our experiment we decided to focus on a surface protein found in chondrocytes, CD44. In previous literature we had found that chondrocytic stem cells that had dedifferentiated into fibroblasts had a large concentration of CD44 surface receptor. We therefore hypothesized that knocking out the CD44 receptor could possibly prevent dedifferentiation of adult chondrocytes. We went about knocking out CD44 with an antibody and then observed collagen I and II levels with transcript and protein level assays. Higher collagen I production is indicative of dedifferentiation, while higher collagen II production suggests the opposite, that chondrocytes are normally functioning.

Method Specifics

We grew chondrocytes on 1% alginate beads. In total, our beads were made from around 4 mL of solution containing 2.5 millions cells/mL. Bovine CD44 antibody was then added to half of the beads. For RNA analysis, we used 66,000 cells for the control and 33,000 cells for the experimental group. Since we could not obtain a measurement for RNA concentration, we used the maximum 20 uL of each sample for the RT-PCR step. Also when we ran the agarose gel during transcript level analysis, we used 18 uL of sample instead of just 10, because we were uncertain how much PCR product we had. The photograph of the gel had an exposure time of .48 seconds. For protein analysis, we used 8 beads for the control group and 3 beads for the experimental group.

Cell Viability Assay

Cells were observed in alginate beads using fluorescence microscopy (Figure 1). We could only observe the cells form the control group, because in our preparation of the experimental group cells for microscopy, they dissolved. Chondrocytes did not seems to exhibit any growth in the core of the beads (Fig. 1A), but were observed clustering together on the surface (Fig. 1B), although they still are very scarce. Differentiating between cells and debris is also a challenge. Furthermore, any cells seemed to also be possibly dead (Fig. 1C). We attempted to use ImageJ to determine the amount of cells, but the program was not able to find anything, because the image is poor and more importantly, there didn't seem to be any clear cells to find.

Transcript Level Analysis – RT-PCR and Gel Electophoresis

After running RT-PCR on the lysates of the cells and amplifying collagen I and II, we ran an electrophoresis gel of the PCR fragments (Figure 2). All of the bands were very faint and barely distinguishable. Even the ladder was blurry and did not seem to show any

bands at the smaller bp sequences. Using ImageJ analysis (Table 1), we determined that collagen I production seemed to go up 20 percent while collagen II production decreased 20 percent.

Protein Level Analysis – ELISA

Following indirect ELISA, we regressed the absorbances of the standards against the concentrations of the standards, and then used that information to determine the concentration of collagen in each of our samples (Figure 3). It appeared to us that Collagen I increased five fold between the experimental and control samples, which is much more than suggested by the transcript level analysis of a 20 percent increase, but nonetheless an increase. The assay for Collagen 2 did not return any results, as no Collagen 2 was detected.

Discussion

The results from our assays suggest that blocking CD44 in adult chondrocytes stimulates their dedifferentiation back into fibroblasts. The transcript level analysis showed that introduction of CD44 antibody seemed to decrease the production of Collagen II and increase the production of Collagen, representative of dedifferentiation. Furthermore, ELISA, seems to back up the result of higher Collagen I concentration. It appears that knocking out CD44 receptor in chondrocytes does not halt dedifferentiation, but on the other hand promotes it.

However, there are several issues in this experiment that need to be addressed. First of all, we do not know if our antibody was a blocking antibody; we assumed it was. Second, our cell viability assay underscores the fact that most of our cells died in the alginate gel, in both the experimental and control groups. This has consequences in our

further assays, because had very little RNA and protein to work with for our RT-PCR and ELISA steps. The absorbance assay prior to RT-PCR confirmed this issue: it perceived no noticeable RNA in the cell lysates, causing us to have to run the RT-PCR with the maximum lysate we could. This also didn't allow us to know how much RNA we started with. This is further highlighted with the long exposure time we had on our gel photograph, suggesting that there was not much DNA to photograph. This also could have affected the ELISA assay, and may be the reason why the Collagen II assay was inconclusive.

The dedifferentiation results are confounding because they are the opposite of what we expected and what is observed in stem cells. The type of antibody we used could have possibly caused these results. Future experiments should repeat this type of study, but instead of knocking down CD44 with antibodies, CD44 should instead be knocked down with RNA interference. Furthermore, it would be interesting to research why culture with CD44 antibody was so fragile, compared to culture without CD44 antibody.

References

Yoshida M, Yasuda T, Hiramitu T, Ito H, Nakamura T. Induction of apoptosis by anti-CD44 antibody in human chondrosarcoma cell line SW1353. Biomed Res. Feb 2008; 29(1):47-52.

Hoben GM, Willard VP, Athanasiou KA. Fibrochondrogenesis of hESCs: growth factor combinations and cocultures. Stem Cells Dev Mar 2009; 18(2):283-92.

A B C

Figure 1. Cell Viability Assay. Cells were stained with STYO10 (green) and ethidium homodimer-2 (red) using the LIVE/DEAD kit and inspected under a fluorescence microscope at 10x. (A) Cells from the core of a bead are shown under green fluorescence. (B,C)Cells from the surface of a bead are shown under green (B) and red (C) fluorescence.

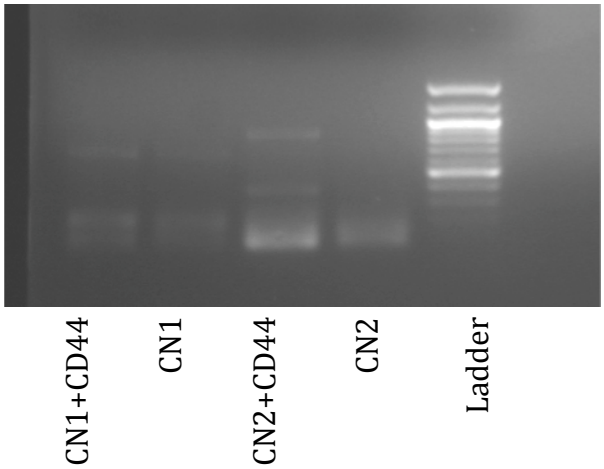

Figure 2. Gel Electophoresis of PCR fragments following RT-PCR. RNA RT-PCR fragments were run through gel electrophoresis in a 1.2% agarose gel at 125V for 45 minutes, and then photographed with an exposure time of .48 s. Samples run are CNI control, CNI+CD44, CNII control, and CNII+CD44.

Ratios	CD44	Control
CN1/GAPDH	2.435897	1.99322
CN2/GAPDH	0.660108	0.855944

Table 1. Ratios of collagen fluorescence to GAPDH fluorescence from gel electrophoresis. Analysis was done with ImageJ.

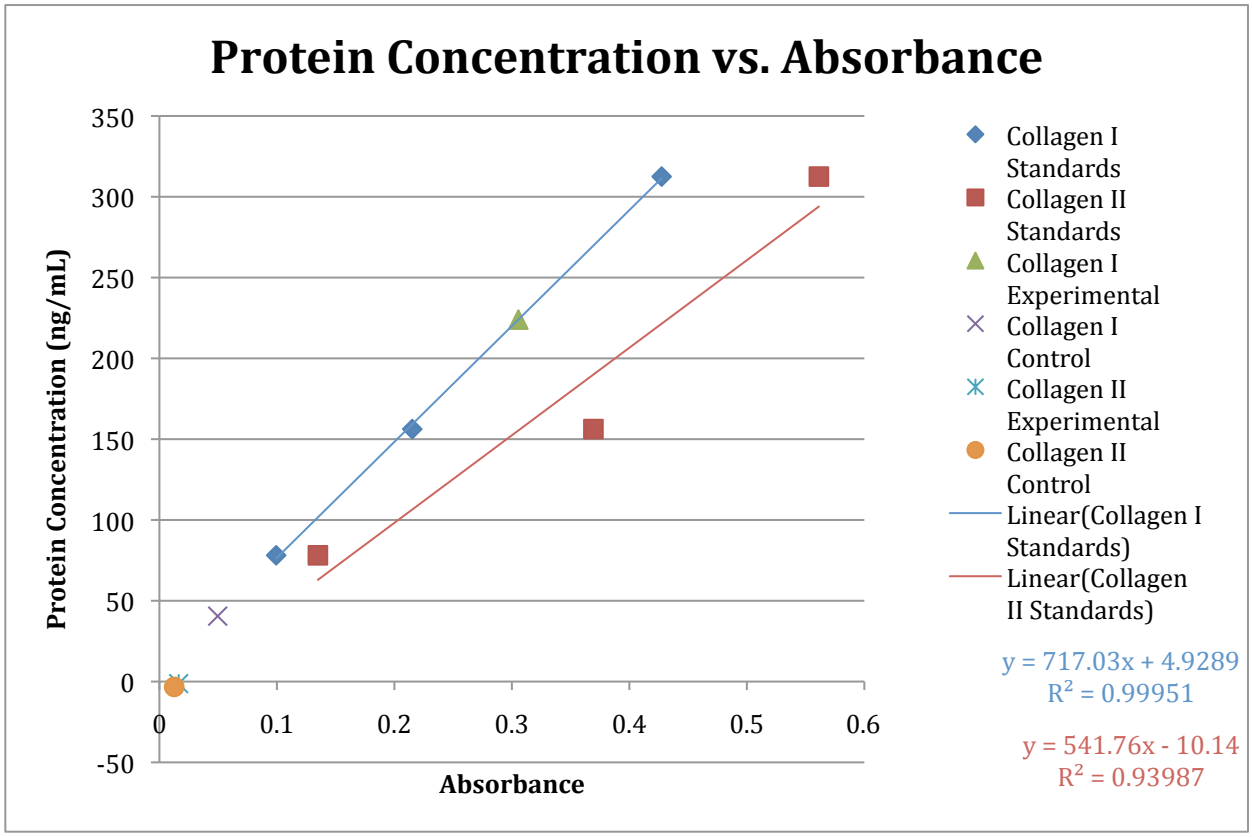

Figure 4. Concentration of collagen I and II from ELISA. ELISA ran at 26.4˚C to detect type 1 and type 2 collagen absorbance in samples containing CD44 and compared to a standard gradient curve (the line of best fit and its equation is shown). Through this analysis we were able to see that CN1 was more highly expressed in samples containing CD44 whereas both CD44+ and CD44- results showed a lack of significant results for CN2 concentration.